Toxicon 93 (2015) 90–97

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

PPAR-γ activation by *Tityus serrulatus* venom regulates lipid body formation and lipid mediator production

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ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form
31 October 2014
Accepted 13 November 2014
Available online 14 November 2014

Keywords:
Tityus serrulatus venom
Lipid bodies
Prostaglandins
Leukotrienes
Toll-like receptors
Peroxisome proliferator-activated receptor gamma

ABSTRACT

Tityus serrulatus venom (TsV) consists of numerous peptides with different physiological and pharmacological activities. Studies have shown that scorpion venom increases pro-inflammatory cytokine production, contributing to immunological imbalance, multiple organ dysfunction, and patient death. We have previously demonstrated that TsV is a venom-associated molecular pattern (VAMP) recognized by TLRs inducing intense inflammatory reaction through the production of pro-inflammatory cytokines and arachidonic acid-derived lipid mediators prostaglandin (PG)E_2 and leukotriene (LT)B_4. Lipid bodies (LBs) are potential sites for eicosanoid production by inflammatory cells. Moreover, recent studies have shown that the peroxisome proliferator-activated receptor gamma (PPAR-γ) is implicated in LB formation and acts as an important modulator of lipid metabolism during inflammation. In this study, we used murine macrophages to evaluate whether the LB formation induced by TsV after TLR recognition correlates with lipid mediator generation by macrophages and if it occurs through PPAR-γ activation. We demonstrate that TsV acts through TLR2 and TLR4 stimulation and PPAR-γ activation to induce LB formation and generation of PGE_2 and LTB_4. Our data also show that PPAR-γ negatively regulates the pro-inflammatory NF-κB transcription factor. Based on these results, we suggest that during envenomation, LBs constitute functional organelles for lipid mediator production through signaling pathways that depend on cell surface and nuclear receptors. These findings point to the inflammatory mechanisms that might also be triggered during human envenomation by TsV.

1. Introduction

Tityus serrulatus (Ts) scorpion causes severe envenomation, specially in infants, who may be at increased risk of death (Amaral et al., 1993). Venoms contain different components capable of activating the immune system through enhanced production of inflammatory mediators (Petricevich, 2010). Indeed, our group has previously shown that Ts venom (TsV) and its toxins Ts1, Ts2, or Ts6 were able to induce the production of nitric oxide (NO), interleukin (IL)-6, and tumor necrosis factor (TNF)-α by macrophages *in vitro* (Zoccal et al., 2011). Besides cytokine production, we have also shown that intraperitoneal injection of Ts2 and Ts6 in mice induced the production of the arachidonic-acid derived inflammatory lipid mediators prostaglandin (PG)E_2 and leukotriene (LT)B_4 (Zoccal et al., 2013). The production of these pro-inflammatory mediators involved the activation of MAPKs and NF-κB dependent pathways through the recognition of Toll-like receptors 2 (TLR2) and 4 (TLR4), and of CD14 (Zoccal et al., 2014). Therefore, we proposed the term venom-associated molecular pattern (VAMP) to designate venom-

Abbreviations: TsV, *Tityus serrulatus* venom; IL, interleukin; TNF, tumor necrosis factor; PG, prostaglandin; LT, leukotriene; LBs, lipid bodies; TLRs, Toll-like receptors; PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern; VAMP, venom-associated molecular pattern; PPAR-γ, peroxisome proliferator-activated receptor gamma; AA, arachidonic acid.

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http://dx.doi.org/10.1016/j.toxicon.2014.11.226
0041-0101/

components that are recognized by receptors of the innate immunity (Zoccal et al., 2014).

LTs, PGs, thromboxanes, and lipoxins are a group of biologically active oxygenated fatty acids known as eicosanoids. Eicosanoids function as paracrine mediators of inflammation as well as intracellular signals (Henderson, 1994; Simchowitz et al., 1994). PGE_2 and LTB_4 are AA-derived metabolites from pathways dependent on cyclooxygenase 1 and 2 (COX-1 and COX-2) and 5-lipoxygenase (5-LO), respectively (Funk, 2001; Samuelsson, 2000). Although the enzymatic pathways for eicosanoid formation are well understood, the intracellular sites of action of these enzymes and the cellular sources of arachidonic acid remain less clear. Recent studies have focused on the intracellular localization of eicosanoid forming enzymes. COXs are associated with cellular membranes, including the endoplasmic reticulum and nuclear membrane (Morita et al., 1995; Otto and Smith, 1994; Smith et al., 1996). In contrast, 5-LO has been localized to the cytoplasm, the perinuclear membrane, and the euchromatin within the nucleus, according to the cell and its state of activation (Brock et al., 1994; Reid et al., 1990; Rouzer and Kargman, 1988; Woods et al., 1995, 1993). While translocation from cytosol to membranes may facilitate interactions of cytosolic enzymes with membrane-bound arachidonate, there is increasing evidence that specific compartmentalization of eicosanoid formation within cells may relate to the different autocrine and paracrine functions of eicosanoids (Almeida et al., 2014; Bozza and Viola, 2010; Serhan et al., 1996; Sorgi et al., 2009). Lipid bodies (LBs) may represent novel potential sites for paracrine eicosanoid production within inflammatory cells.

LBs are intracellular lipid organelles, found in different cell types, including macrophages, which participate in lipid metabolism, inflammatory mediator production, membrane trafficking, and intracellular signaling (Bozza et al., 2007; Martin and Parton, 2006). LBs have emerged as compartmentalization sites for the metabolism of lipid mediators, especially in inflammatory and infectious conditions (Bozza et al., 2011; Pacheco et al., 2002; Vieira-de-Abreu et al., 2005). Studies have showed that increased amounts of LB correlated with the increased release of LTB_4, LTC_4, and PGE_2 by leukocytes activated with the calcium ionophore A23187 (Bandeira-Melo et al., 2001; Bozza et al., 1996, 1997a, 1997b; Pacheco et al., 2002). Moreover, our group has previously shown that LB formation induced by *Histoplasma capsulatum* is associated with LTB_4 and PGE_2 production (Sorgi et al., 2009). However, the potential role of LBs as sites for lipid mediator production induced by TsV has not yet been addressed.

Distinct signaling pathways have been suggested to be involved in LB formation, including those that operate downstream of TLRs. LB formation has been shown to be dependent on TLR2/TLR6 in *Mycobacterium leprae*-infected macrophages (de Mattos et al., 2012), or on CD36/TLR2 and peroxisome proliferator-activated receptor gamma (PPAR-γ) in *Mycobacterium bovis* bacillus Calmette Guérin (BCG) infection (Almeida et al., 2014). PPARs are lipid-activated transcription factors that are ligand dependent. These receptors regulate target gene expression by binding to specific peroxisome proliferator responsive elements located in the regulatory sites of each gene. Agonists alter and stabilize the conformation of PPAR-γ, creating a binding site, with subsequent recruitment of transcriptional co-activators, resulting in increased target gene transcription. PPAR-γ, specifically, has been shown to function as a key transcriptional regulator of cell differentiation, inflammation, and lipid metabolism in macrophages and dendritic cells (Szatmari and Nagy, 2008). In *Mycobacterium* infection, the PPAR-γ role in the regulation of lipid metabolism, lipid body formation, and immune response has been well established (Almeida et al., 2012, 2009; de Mattos et al., 2012; Mahajan et al., 2012; Rajaram et al., 2010).

Considering the research findings detailed above, we hypothesized that PPAR-γ activation is involved in LB formation and consequently in lipid mediator production by macrophages in response to TsV-induced TLR stimulation. We showed here that LB formation induced by TsV correlates with enhanced lipid mediator generation by macrophages. We also showed that TsV-elicited LB formation and lipid mediators production are TLR4, TLR2 and PPAR-γ dependent.

2. Materials and methods

2.1. Animals

TLR2 and TLR4 knockout (KO or $^{-/-}$) mice, from 6 to 8 weeks, were donated by S. Akira (Osaka University, Osaka, Japan) and bred in the Animal House of the Faculdade de Medicina de Ribeirão Preto (Universidade de São Paulo, Ribeirão Preto, Brazil). Strain-matched wild type (WT) C57Bl/6 (C57Bl/6 was the genetic background for the $TLR2^{-/-}$ and $TLR4^{-/-}$ animals) mice of both sexes were bred in the Faculdade de Ciências Farmacêuticas de Ribeirão Preto (Universidade de São Paulo, Ribeirão Preto, Brazil). Mice were maintained at 25 °C, with a 12 h light/dark cycle, and with free access to food and water. The experimental protocols were approved by the Animal Care Committee of the Prefeitura of the Campus of Ribeirão Preto (PCARP) at Universidade de São Paulo, Ribeirão Preto, Brazil (Protocol number 11.1.160.53.1).

2.2. Tityus serrulatus venom

TsV was extracted from *T. serrulatus* scorpion as previously described (Arantes et al., 1989), desiccated, and stored at −20 °C. Prior to the experiments, TsV was diluted in phosphate buffered saline (PBS) and filtered through sterilizing membranes (Spritzenfilter: 0.22 μm, TPP, Switzerland). The *Limulus* Amoebocyte Lysate test (LAL) (QCL-1000, Bio Whittaker, Cambrex Company, Walkersville, MD, USA) was performed to detect LPS in TsV samples, according to the manufacturer's instructions.

2.3. Investigation of PPAR-γ participation in LB formation and lipid mediator release

Leukocytes were isolated from the peritoneal cavities of C57Bl/6, $TLR2^{-/-}$ or $TLR4^{-/-}$ mice by lavage with 3 mL of RPMI incomplete medium. After centrifugation at 400× *g*, the cells were plated in 8-well micro culture slides (BD Bioscience) at a density of 2×10^5 cells/well in RPMI medium supplemented with 10 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal bovine serum (FBS). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere for 18 h. After washing with sterile PBS, adherent cells (macrophages) were pretreated or not with PPAR-γ receptor antagonist (GW9662, 10 μM, Cayman Chemical, USA) in fetal bovine serum (FBS)-free RPMI for 30 min and stimulated or not with TsV (50 μg/mL) for 0.5, 4 or 24 h at 37 °C in a 5% CO_2 atmosphere. After stimulation, cell free supernatants were harvested and stored at −20 °C for cytokine quantification (item 2.4) and cells were prepared for LB evaluation (item 2.5) or to maximal PGE_2 and LTB_4 release (item 2.6). For quantification of lipid mediators, adherent macrophages were suspended in $Ca^{2+}Mg^{2+}$ HBSS and stimulated for 15 min with 0.5 μM calcium ionophore A23187 (Sigma–Aldrich).

2.4. Cytokine measurements

Cell free supernatants of adherent macrophages from C57Bl/6 mice pretreated or not with GW9662 for 30 min and stimulated or not with TsV (50 μg/mL) for 24 h were collected and concentrations

of IL-6, IL-1β, and KC were quantified by enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). The optical densities were measured at 405 nm in a microplate reader (μQuant, Biotek Instruments Inc.). The sensitivities were >10 pg/mL.

2.5. LB staining and quantification

Macrophages adhered to slides were fixed in 3.7% formaldehyde in Ca^{+2}/Mg^{+2}-free HBSS (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), and stained in 1.5% osmium tetroxide (30 min). After being rinsed in water, cells were immersed in 1.0% thiocarbohydrazide (5 min), rinsed in water and then in 0.1 M cacodylate buffer, incubated in 1.5% osmium tetroxide (3 min), rinsed in distilled water, dried, and mounted in slides for further analysis. The morphology of fixed cells was observed, and LBs were quantified by light microscopy with a 100× objective lens in 150 leukocytes that were stimulated in triplicate (50 consecutive leukocytes were analyzed in each slide). Blind observers performed LB quantification.

2.6. LTB_4 and PGE_2 measurements

LTB_4 and PGE_2 concentrations in the supernatants were assayed by a specific enzyme immunoassay (Cayman Chemical, USA), according to the manufacturer's instructions. Sample absorbance was measured at 420 nm in a microplate reader (μQuant, Biotek Instruments Inc.), and the concentrations of eicosanoids were calculated based on the standard curve. The detection limit was >13 pg/mL.

2.7. NF-kB reporter assay

RAW-Blue™ cells, RAW264.7 macrophages that stably express the secreted embryonic alkaline phosphatase (SEAP) gene, which is inducible by the NF-κB/AP-1 transcription factors and are resistant to the selectable marker Zeocin, were donated by Dr. Huy Ong (Université de Montréal, Canada). The cells were seeded in 96-well micro culture plates at a density of 2×10^5 cells/well in DMEM supplemented with Normocin™ (50 mg/mL) and cultured at 37 °C in a humidified 5% CO_2 atmosphere for 18 h. After this period, the cells were incubated with the PPAR-γ antagonist (GW9662, 10 μM, Cayman Chemical, USA) or with the PPAR-γ agonist Roziglitazone (Rz, 80 μM, Cayman Chemical, USA) for 30 min and then stimulated with TsV (50 μg/mL) for 24 h. After this period, the medium was collected, and 50 μL samples were mixed, in 96-well plates at 37 °C for 2 h, with 150 μL of QUANTI-Blue™ (InvivoGen, USA), which is a SEAP detection medium. The optical density was then measured at 650 nm using an ELISA reader (μQuant, Biotek Instruments Inc.).

2.8. Gene expression analysis by real-time PCR (qRT-PCR)

Macrophage mRNA expression was evaluated by qRT-PCR after 0.5, 4, and 24 h of stimulation with TsV. mRNA was isolated using the RNeasy Mini kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. cDNA (complementary DNA) was synthesized from 200 ng of total RNA using random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Temecula, CA). Aliquots of 2 μl of total cDNA were amplified by qRT-PCR (StepOne Plus, Applied Biosystems) using the primers and probe for *Pparg* (Mm01184322) (TaqMan® Gene Expression Assay, Applied Biosystems). *Gapdh* (Mm99999915) and *Actb* (Mm00607939) were used as reference genes. Amplification was performed under the following conditions: denaturation at 95 °C for 10 min; followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Relative quantification was performed using the ΔΔCt Method.

2.9. Statistical analysis

Data are expressed as means ± SEM and were analyzed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. TsV induces LB formation, and LTB_4 and PGE_2 production in macrophages

Based on our previous results showing that 50 μg/mL of TsV did not affect peritoneal macrophage viability (Zoccal et al., 2014), we used this same concentration for cell stimulation. We first investigated the TsV capacity to induce LB formation in peritoneal macrophages. As shown in Fig. 1(A), TsV increased LB formation in peritoneal macrophages after 0.5, 4, and 24 h of stimulation. Because LBs are specialized organelles involved in amplification of lipid mediator synthesis (Bozza and Viola, 2010) we next investigated whether the increased numbers of LBs in TsV-stimulated macrophages would correlate with increased LTB_4 and PGE_2 production by these cells. Indeed, macrophages exposed to TsV and stimulated with the calcium ionophore A23187 increased LTB_4 production in a pattern that very closely matched LB formation (Fig. 1(B)). PGE_2 production also increased at all time points compared to non-stimulated controls, albeit with a different pattern (Fig. 1(C)). These results indicate that LBs are important for lipid mediator generation during envenomation.

3.2. TsV induces LB formation through PPAR-γ via, TLR2 and TLR4

Because TLRs have been implicated in LB formation (Sorgi et al., 2009), we used peritoneal macrophages from $TLR2^{-/-}$ and $TLR4^{-/-}$ mice to investigate the role of these TLRs in LB formation. We observed decreased LB formation in TLR2-deficient macrophages stimulated with TsV for 0.5, 4, and 24 h, and in TLR4-deficient macrophages after 0.5 and 4 h of stimulation (Fig. 2(A)). Given the involvement of the transcription factor PPAR-γ in LB formation through TLR2 recognition (Almeida et al., 2014), our next step was to evaluate *Pparg* expression in TsV-stimulated macrophages. qRT-PCR analysis revealed that TsV up-regulated *Pparg* mRNA expression at 4 and 24 h stimulation (Fig. 2(B)). We also evaluated *Pparg* expression in TLR2 and TLR4 deficient macrophages by qRT-PCR after 24 h of TsV stimulation. TsV up-regulated the expression levels of *Pparg* in macrophages from C57Bl/6 mice, whereas in cells from $TLR2^{-/-}$ and $TLR4^{-/-}$ mice up-regulation also occurred but to a significantly lower extent (Fig. 2(C)). To further investigate the role of PPAR-γ in LB formation, we pretreated macrophages with the PPAR-γ antagonist GW9662 and then stimulated the cells with TsV. LB formation in cells treated with GW9662 after 4 and 24 h of TsV stimulation was not significantly greater than in non-TsV-stimulated controls (Fig. 2(D)). Altogether, these results indicate that TLR2, TLR4, and PPAR-γ regulate LB formation in TsV-stimulated macrophages.

3.3. PPAR-γ activation inhibits NF-κB in TsV-stimulated macrophages

To confirm that PPAR-γ also serves as a negative regulator of macrophage differentiation and activation through transrepression of the transcription factors (Ricote et al., 1998), we investigated NF-κB expression in TsV-stimulated cells pretreated with a PPAR-γ antagonist or an agonist. Our data show that the PPAR-γ agonist Rz significantly inhibits NF-κB activation (Fig. 3). Rz has the potential to exert biological activities independent of PPAR-γ activation (Neri et al., 2011), thus we sought to confirm the role of PPAR-γ activation

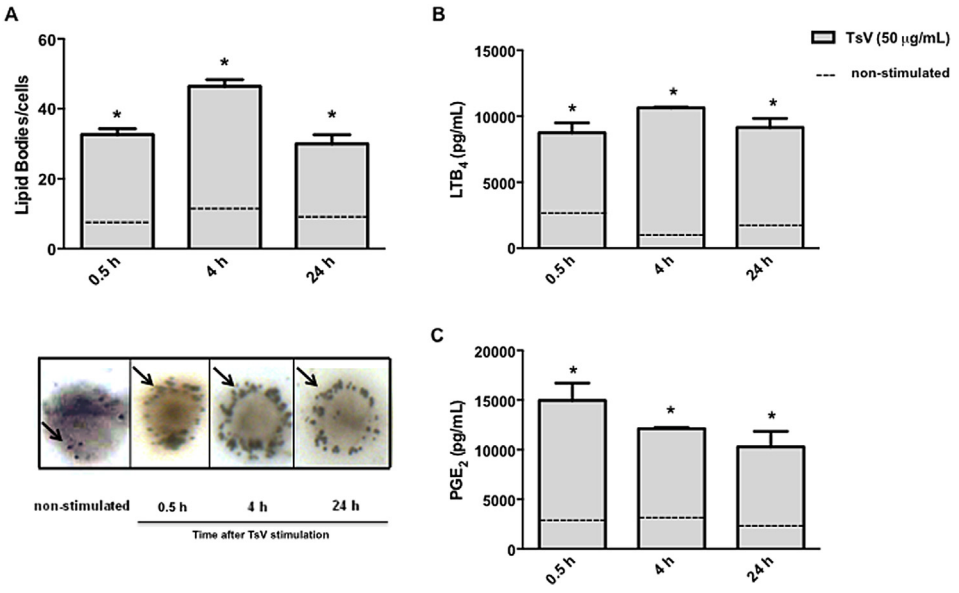

Fig. 1. TsV induces lipid body formation and PGE_2 and LTB_4 production. Adherent macrophages from C57Bl/6 mice were stimulated with TsV (50 μg/mL) for 0.5, 4, and 24 h. Non-stimulated cells were used as control. Cells were stained with osmium tetroxide for LB analysis (A), or stimulated for 15 min with A23187 (0.5 μM) for quantification of LTB_4 (B) and PGE_2 (C). Results are expressed as means ± SEM ($n = 4$), and are from 3 independent experiments ($n = 12$). LBs per cell were enumerated by light microscopy analysis with a 100× objective lens. $^*p < 0.05$ (one-way ANOVA) compared to control (dashed line).

in the modulation of TsV-induced peritoneal macrophages using the specific PPAR-γ inhibitor GW9662. The inhibitory effects were reverted by the specific antagonist demonstrating the negative role of PPAR-γ in NF-κB activation (Fig. 3).

3.4. PPAR-γ is involved in LTB_4 and PGE_2 production by TsV-stimulated macrophages

We previously demonstrated, *in vitro*, that PGE_2 and LTB_4 are produced upon TsV stimulation of macrophages (Zoccal et al.,

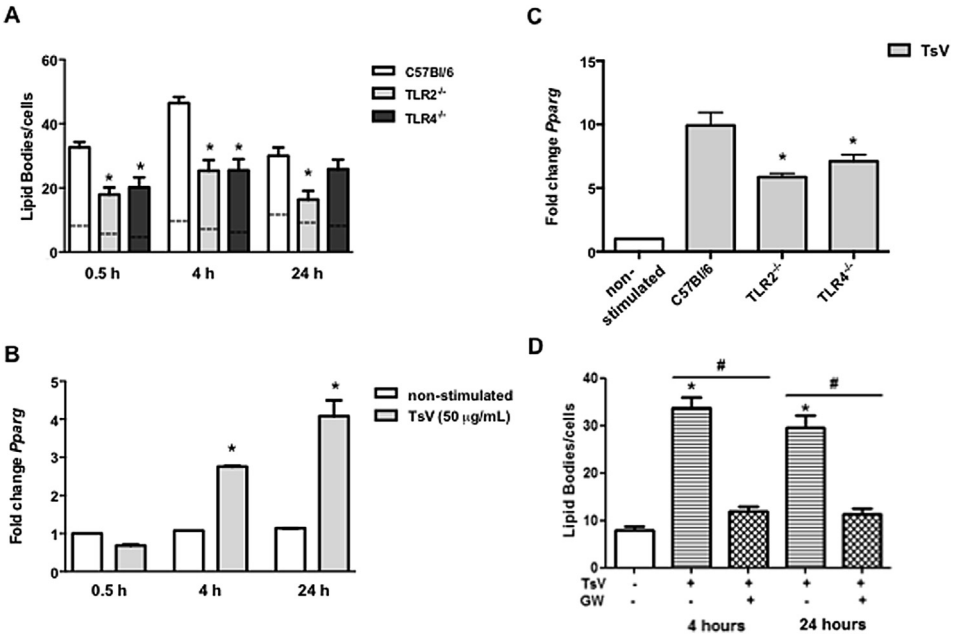

Fig. 2. Lipid body formation induced by TsV involves TLR4 and TLR2 recognition and PPAR-γ expression. (A) Peritoneal macrophages from C57Bl/6, $TLR2^{-/-}$ or $TLR4^{-/-}$ mice were stimulated with TsV (50 μg/mL) for 0.5, 4, and 24 h. LBs were counted in peritoneal macrophages after osmium tetroxide staining. Results are expressed as means ± SEM ($n = 3$), and were obtained from 2 independent experiments ($n = 6$). $^*p < 0.05$ (one-way ANOVA) compared to medium alone (dashed line). Adherent macrophages from C57Bl/6 (WT) mice were treated with TsV (50 μg/mL) for 0.5, 4 or 24 h (B) and cells from $TLR2^{-/-}$ or $TLR4^{-/-}$ were treated with TsV (50 μg/mL) for 24 h (C). Non-stimulated macrophages were used as the negative control. Cells were lysed, and total RNA was extracted. qRT-PCR was performed to determine the relative expression levels of transcripts encoding *Pparg*. Results were normalized to the expression levels of the reference genes *Actb* and *Gapdh*. The ΔΔCt method was used for the analysis of qRT-PCR data. $^*p < 0.05$ (one-way ANOVA followed by Dunnett's post-test) compared to control. Differences were considered statistically significant when $p < 0.05$. (D) Adherent macrophages from C57Bl/6 mice were pretreated or not with the PPAR-γ antagonist GW9662 (10 μM) 30 min before stimulation with TsV (50 μg/mL) for 4 and 24 h. Non-stimulated cells were used as the negative control. LBs per cell were enumerated by light microscopy analysis with a 100× objective lens. Results are expressed as means ± SEM ($n = 4$), and were obtained from 2 independent experiments ($n = 8$). $^*p < 0.05$ (one-way ANOVA) compared to control and $^\#p < 0.05$ compared to TsV.

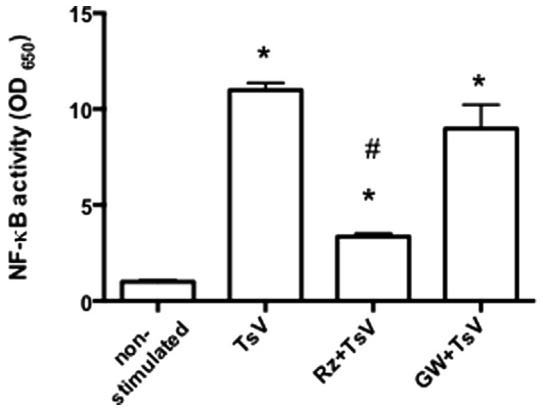

Fig. 3. Activation of NF-κB/AP-1 in RAW-Blue™ cells. These cells were derived from RAW 264.7 macrophages and contain a secreted embryonic alkaline phosphatase (SEAP) reporter construct that is integrated into the cellular DNA and that can be induced by NF-κB. The cells were incubated with PPAR-γ antagonist (GW9662, 10 μM) or with PPAR-γ agonist (Rz, 80 μM) for 30 min and then stimulated with TsV (50 μg/mL) for 24 h. After 24 h of stimulation, the QUANTI-Blue™ substrate was used to measure the SEAP at 650 nm with an ELISA reader. $^{*}p < 0.05$ (one-way ANOVA) compared to non-stimulated cells; $^{\#}p < 0.05$ compared to TsV. Results are expressed as means ± SEM ($n = 4$) and were obtained from 2 independent experiments ($n = 8$).

2014). To further investigate if production of lipid mediators after TsV stimulation plus calcium ionophore A23187 is TLR2 and TLR4 dependent, we quantified LTB_4 in stimulated and non-stimulated macrophages from $TLR2^{-/-}$ or $TLR4^{-/-}$ mice. For this, peritoneal macrophages were stimulated with TsV or not for 4 h. After this period, adherent macrophages were suspended in $Ca^{2+}Mg^{2+}$ HBSS and stimulated with calcium ionophore A23187. TsV-induced LTB_4 production was totally impaired in $TLR2^{-/-}$ and $TLR4^{-/-}$ cells (Fig. Supplementary 1). This result is similar to our previous study (Zoccal et al., 2014), however the magnitude is different, demonstrating that, in the presence of the calcium ionophore, lipid mediators production is enhanced. We next investigated the involvement of PPAR-γ in LTB_4 and PGE_2 production in response to TsV stimulation. Pretreatment of macrophages with GW9662 significantly inhibited the production of these lipid mediators (Fig. 4(A) and (B), respectively). On the other hand, GW9662 did not affect the production of protein inflammatory mediators IL-6, IL-1β, and KC induced by TsV (Fig. 4(C)–(E), respectively). Our results indicate that PPAR-γ signaling pathways are activated upon TsV recognition through TLRs and play an important role in LB formation and lipid mediator production.

4. Discussion

In this study, we show that TsV recognition through TLR2 and TLR4 activates PPAR-γ to regulate LB formation, as well as lipid metabolism in macrophages, indicating that PGE_2 and LTB_4 generation during envenomation is dependent on LB formation.

Inflammatory mediators are released when pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or more recently VAMP are recognized by pattern recognition receptors (PRRs) in immune cells (Murray and Wynn, 2011; O'Neill and Bowie, 2007; Yang et al., 2013; Zoccal et al.,

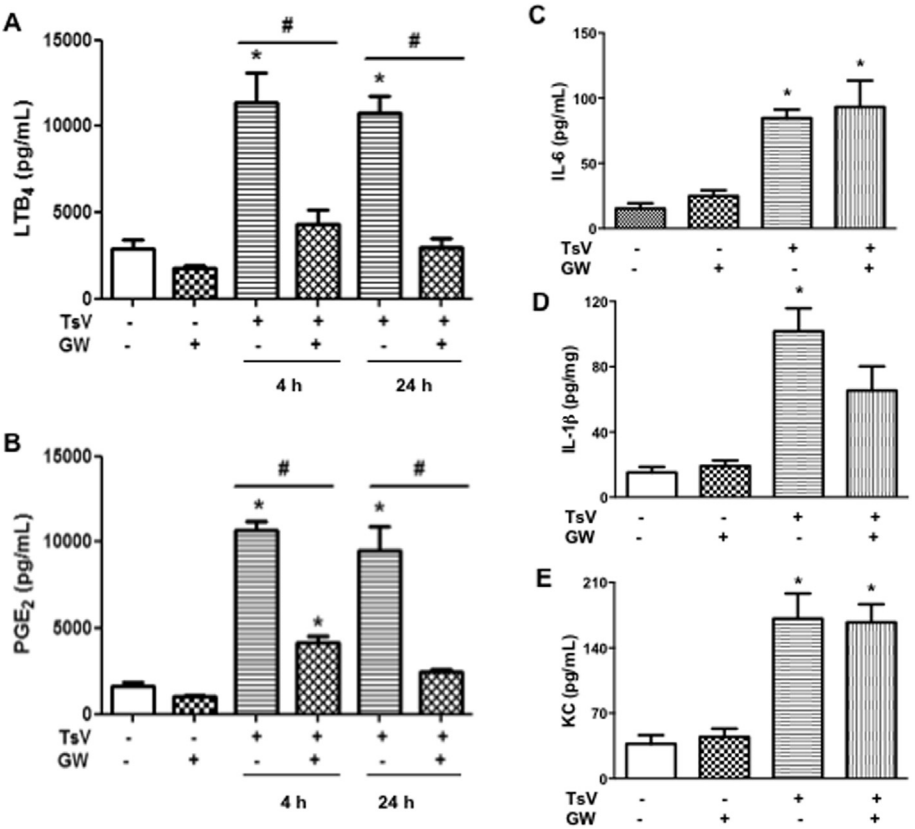

Fig. 4. PPAR-γ acts as a key modulator of lipid metabolism. Adherent macrophages from C57Bl/6 mice were pretreated or not with GW9662 (10 μM) 30 min before stimulation with TsV (50 μg/mL) for 4 and 24 h. Unstimulated macrophages were used as the negative control. Macrophages were stimulated for 15 min with A23187 (0.5 μM) for quantification of LTB_4 (A) and PGE_2 (B) production $^{*}p < 0.05$ (one-way ANOVA) compared to non-stimulated cells and $^{\#}p < 0.05$ compared to TsV. Adherent cells were stimulated with TsV (50 μg/mL) for 24 h, and IL-6 (C), IL-1β (D), and KC (E) production was measured by ELISA. Results are expressed as means ± SEM ($n = 4$), and were obtained from 2 independent experiments ($n = 8$). $^{*}p < 0.05$ (one-way ANOVA) compared to non-stimulated cells.

2014). Our group also has shown that TLR2 is involved in macrophage LB formation induced by *H. capsulatum* infection (Sorgi et al., 2009). Likewise, LB formation in LPS-stimulated macrophages has been shown to be dependent on TLR4 (Pacheco et al., 2002). Therefore, to clarify the role of PRR in TsV-induced LB formation, we investigated the possible participation of TLR. LB formation was impaired in TLR2$^{-/-}$ and TLR4$^{-/-}$ macrophages, indicating an important role for these receptors in the LB biogenesis pathway. We speculate that TsV-induced LB formation occurs through either: (1) direct interactions between the venom and cell receptors, or (2) an indirect mechanism mediated by venom-induced cytokines and chemokines.

Our results suggest that LB formation through TLR2/TLR4 signaling might be important to LTB_4 production, whereas PGE_2 production has previously been shown to depend on TLR4 but not TLR2 (Zoccal et al., 2014). Our previous study demonstrated that TsV *per se* induces lipid mediators (Zoccal et al., 2014) and we confirmed that here using a calcium ionophore as an unspecific stimulus known to potentiate lipid mediator release by cells (Rao et al., 1993). Here, we also show that TsV-induced cell signaling generates lipid mediators in LB-dependent manner. However, other mechanisms might be involved in venom recognition to induce lipid mediators, thus, other cellular activation pathways, and the correlation between lipid mediator production and LB formation still require further elucidation.

Besides lipid storage and metabolism, LBs may function in membrane trafficking and cell signaling (Fujimoto et al., 2004; Liu et al., 2004; Wan et al., 2007), and in compartmentalization of different classes of proteins, such as caveolin, proteins of the Rab family (Liu et al., 2004; Martin et al., 2005; Pol et al., 2004; Wan et al., 2007), eicosanoid-forming enzymes (Bozza et al., 1998; Pacheco et al., 2002), and protein kinases like PI3 kinase, MAP kinase, and PKC (Chen et al., 2002; Yu et al., 1998, 2000). Studies have shown that LB biogenesis is strictly controlled (Bozza et al., 2007). PKC, PI3K, ERK1/2, and $iPLA_2$ signaling pathways have been implicated in LB formation induced by Lys49 phospholipase A_2 from *Bothrops asper* snake venom (Giannotti et al., 2013). Our results indicate that PPAR-γ is involved in TsV-induced LB formation and PGE_2 and LTB_4 production by macrophages, which were inhibited by the PPAR-γ antagonist. These results agree with those of a previous study that showed that a PPAR-γ antagonist inhibited LB biogenesis in *M. bovis* BCG-infected macrophages (Almeida et al., 2014). On the other hand, PGE_2 release by GM-CSF-primed bone marrow-derived macrophages has been shown to be independent of PPAR-γ activation, but dependent on TLR2 and its co-receptors TLR1, TLR6, and CD14, as well as on MyD88 and NF-κB (Almeida et al., 2012; Sorgi et al., 2012). PPAR-γ expression in macrophages infected with BCG or stimulated with ManLAM is dependent on TLR2 signaling (Almeida et al., 2009). However, the nonpathogenic *Mycobacterium smegmatis*, a well-known TLR2 ligand, and the synthetic TLR2 ligand Pam3Cys fail to induce PPAR-γ expression in macrophages (Almeida et al., 2009; Rajaram et al., 2010), suggesting that co-receptors of TLR2 are required to induce PPAR-γ expression. We know that PPAR-γ also serves as a negative regulator of macrophage activation, altering the expression of several inflammatory genes (Jiang et al., 1998; Ricote et al., 1998), modulating macrophage differentiation and activation through transrepression of the transcription factors STAT, AP-1, and NF-κB (Ricote et al., 1998), and attenuating the respiratory burst (Von Knethen and Brune, 2002).

Altogether, our results suggest a central role for LBs as compartmentalization sites for lipid mediator generation in cells involved in envenomation. Considering our previous results (Zoccal et al., 2014) and those obtained in this study, we propose a model in which TsV recognition through macrophage TLR4/TLR2 activates NF-κB and/or PPAR-γ. PPAR-γ activation by TsV would result in LB formation, PGE_2 and LTB_4 production, while NF-κB activation would result in pro-inflammatory cytokine production (Fig. 5). We hypothesize that according to the stimuli, LBs compartmentalize different sets of proteins and their metabolic products can lead either to pro-inflammatory amplification or to down-modulation of leukocyte functions. This model is consistent with a previous study showing that in mycobacterial infection, PPAR-γ activation can negatively modulate NF-κB activation, inhibiting the production of pro-inflammatory cytokines by macrophages (Almeida et al., 2012). Although PPAR-γ downstream effectors and target genes activated by TsV remain to be elucidated, PPAR-γ can be considered an attractive target candidate for therapeutic intervention strategies that is able to diminish the damage caused by *T. serrulatus* venom.

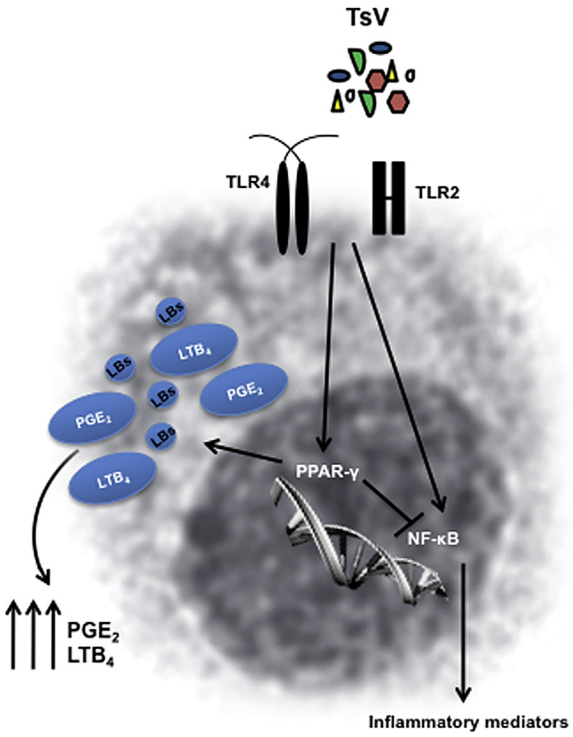

Fig. 5. TsV recognition through TLR4/TLR2 results in PPAR-γ and NF-κB activation. TsV-induced production of inflammatory mediators by macrophages can occur in two ways: (1) TsV induces NF-κB activation that results in inflammatory mediator release, or (2) TsV induces PPAR-γ activation that results in LB formation and, consequently, in increased PGE_2 and LTB_4 production. Interestingly, TsV-induced PPAR-γ activation suppress NF-κB signaling pathway.

Ethical statement

We certify that human subjects were not used in this work. TLR2 and TLR4 knockout (KO or −/−) mice between the ages of 6 and 8 weeks were donated by S. Akira (Osaka University, Osaka, Japan) and bred in the Animal House of the Faculdade de Medicina de Ribeirão Preto (Universidade de São Paulo, Ribeirão Preto, Brazil). Strain-matched WT C57Bl/6 (C57Bl/6 genetic background for the TLR2 and TLR4) mice of both sexes were bred in the Faculdade de Ciências Farmacêuticas de Ribeirão Preto (Universidade de São Paulo, Ribeirão Preto, Brazil). The mice were maintained in a room at 25 °C with a light/dark cycle of 12 h and provided with free access to food and water. Mice were kept in biohazard facilities throughout the experiments, which were approved by the Animal Care Committee of the Prefeitura of the Campus of Ribeirão Preto (PCARP) at the University of São Paulo (Protocol number 11.1.160.53.1).

Acknowledgments

We are grateful to the São Paulo Research Foundation (FAPESP, grants# 2009/07169-5 and 2011/18179-9) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.11.226.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.11.226.

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